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☐ 1. Document ID: US 6555675 B2

L4: Entry 1 of 6 File: USPT Apr 29, 2003

US-PAT-NO: 6555675
DOCUMENT-IDENTIFIER: US 6555675 B2

TITLE: Dinucleoside polyphosphate compositions and their therapuetic use as purinergic receptor agonists

DATE-ISSUED: April 29, 2003

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Rideout; Janet | Raleigh | NC | | |
| Yerxa; Benjamin R. | Raleigh | NC | | |
| Shaver; Sammy Ray | Chapel Hill | NC | | |
| Douglass, III; James G. | Apex | NC | | |

US-CL-CURRENT: 536/25.6; 424/45, 424/46, 536/26.23

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 6462028 B2

L4: Entry 2 of 6 File: USPT Oct 8, 2002

US-PAT-NO: 6462028
DOCUMENT-IDENTIFIER: US 6462028 B2

TITLE: Method of promoting cervical and vaginal secretions

DATE-ISSUED: October 8, 2002

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Pendergast; William | Durham | NC | | |
| Shaver; Sammy R. | Chapel Hill | NC | | |
| Drutz; David J. | Chapel Hill | NC | | |
| Rideout; Janet L. | Raleigh | NC | | |
| Yerxa; Benjamin R. | Raleigh | NC | | |

US-CL-CURRENT: 514/47; 514/48, 514/50

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw Desc | Image

☐ 3. Document ID: US 6348589 B1

L4: Entry 3 of 6 File: USPT Feb 19, 2002

US-PAT-NO: 6348589
DOCUMENT-IDENTIFIER: US 6348589 B1
**** See image for Certificate of Correction ****

TITLE: Certain dinucleotides and their use as modulators of mucociliary clearance and ciliary beat frequency

DATE-ISSUED: February 19, 2002

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Pendergast; William | Durham | NC | | |
| Yerxa; Benjamin R. | Raleigh | NC | | |
| Rideout; Janet L. | Raleigh | NC | | |
| Siddiqi; Suhaib M. | Raleigh | NC | | |

US-CL-CURRENT: 536/25.6; 536/26.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 4. Document ID: US 6323187 B1

L4: Entry 4 of 6 File: USPT Nov 27, 2001

US-PAT-NO: 6323187
DOCUMENT-IDENTIFIER: US 6323187 B1
**** See image for Certificate of Correction ****

TITLE: Therapeutic dinucleotide and derivatives

DATE-ISSUED: November 27, 2001

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Yerxa; Benjamin R. | Raleigh | NC | | |
| Pendergast; William | Durham | NC | | |
| Rideout; Janet L. | Raleigh | NC | | |
| Picher; Maryse | Carrboro | NC | | |
| Boucher, Jr.; Richard C. | Chapel Hill | NC | | |
| Stutts; M. Jackson | Chapel Hill | NC | | |

US-CL-CURRENT: 514/51; 514/42, 514/43, 514/44, 514/49, 514/52, 536/25.6, 536/26.23

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 5. Document ID: US 6294188 B1

L4: Entry 5 of 6 File: USPT Sep 25, 2001

US-PAT-NO: 6294188
DOCUMENT-IDENTIFIER: US 6294188 B1

TITLE: Methods involving changing the constitutive and stimulated secretions of the local reproductive system of women

DATE-ISSUED: September 25, 2001

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Ragavan; Vanaja V. | Wynnewood | PA | | |
| Laties; Alan | Philadelphia | PA | | |

US-CL-CURRENT: 424/433; 424/430, 424/431, 424/432, 424/434, 424/45, 424/464, 424/489

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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☐ 6. Document ID: US 5837861 A

L4: Entry 6 of 6 File: USPT Nov 17, 1998

US-PAT-NO: 5837861
DOCUMENT-IDENTIFIER: US 5837861 A
**** See image for Certificate of Correction ****

TITLE: Dinucleotides and their use as modulators of mucociliary clearance and ciliary beat frequency

DATE-ISSUED: November 17, 1998

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Pendergast; William | Durham | NC | | |
| Yerxa; Benjamin R. | Raleigh | NC | | |
| Rideout; Janet L. | Raleigh | NC | | |
| Siddiqi; Suhaib M. | Raleigh | NC | | |

US-CL-CURRENT: 536/25.6; 536/26.23

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | KWIC | Draw Desc | Image

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This paragraph must follow form paragraph 23.09 and should only be used in those rare instances where both the patent and the application claim distinct, interfering inventions.

## 2307 Applicant Requests Interference With a Patent

*37 CFR 1.607. Request by applicant for interference with patent.*

(a) An applicant may seek to have an interference declared between an application and an unexpired patent by,

(1) Identifying the patent,

(2) Presenting a proposed count,

(3) Identifying at least one claim in the patent corresponding to the proposed count,

(4) Presenting at least one claim corresponding to the proposed count or identifying at least one claim already pending in its application that corresponds to the proposed count, and, if any claim of the patent or application identified as corresponding to the proposed count does not correspond exactly to the proposed count, explaining why each such claim corresponds to the proposed count, and

(5) Applying the terms of any application claim,

(i) Identified as corresponding to the count, and

(ii) Not previously in the application to the disclosure of the application.

(6) Explaining how the requirements of 35 U.S.C. 135(b) are met, if the claim presented or identified under paragraph (a)(4) of this section was not present in the application until more than one year after the issue date of the patent.

(b) When an applicant seeks an interference with a patent, examination of the application, including any appeal to the Board, shall be conducted with special dispatch within the Patent and Trademark Office. The examiner shall determine whether there is interfering subject matter claimed in the application and the patent which is patentable to the applicant subject to a judgment in an interference. If the examiner determines that there is any interfering subject matter, an interference will be declared. If the examiner determines that there is no interfering subject matter, the examiner shall state the reasons why an interference is not being declared and otherwise act on the application.

(c) When an applicant presents a claim which corresponds exactly or substantially to a claim of a patent, the applicant shall identify the patent and the number of the patent claim, unless the claim is presented in response to a suggestion by the examiner. The examiner shall notify the Commissioner of any instance where an applicant fails to identify the patent.

(d) A notice that an applicant is seeking to provoke an interference with a patent will be placed in the file of the patent and a copy of the notice will be sent to the patentee. The identity of the applicant will not be disclosed unless an interference is declared. If a final decision is made not to declare an interference, a notice to that effect will be placed in the patent file and will be sent to the patentee.

If the applicant does not apply the terms of the claim presented to the disclosure of the application, i.e., does not state how each term of the copied claim is supported by the specification, as required by 37 CFR 1.607(a)(5), a one-month time period should be set for correction of this deficiency. Form Paragraph 23.12 should be used for this purpose.

### COMPLIANCE WITH 35 U.S.C. 135(b)

If the claim presented or identified as corresponding to the proposed count was added to the application by an amendment filed more than one year after issuance of the patent, or the application was not filed until more than one year after issuance of the patent (but the patent is not a statutory bar), then under the provisions of 35 U.S.C. 135(b), an interference will not be declared unless at least one of the claims which were in the application, or in a parent application, prior to expiration of the one-year period was for "substantially the same subject matter" as at least one of the claims of the patent. Therefore, 37 CFR 1.607(a)(6) requires that the request for interference with the patent include an explanation of how the requirements of 35 U.S.C. 135(b) are met. If this explanation is not provided, a one-month time period should be set for correction of this deficiency.

¶ *23.09 Requirement To Copy Patent Claim*

The following claim number **[ 1 ]** from U.S. Patent No. **[ 2 ]** is suggested to applicant under 35 U.S.C. 135(a) for the purposes of an interference:

**[ 3 ]**

The suggested claim must be copied exactly, although other claims may be proposed under 37 CFR 1.605(a).

Applicant is given ONE MONTH or THIRTY DAYS, whichever is longer, from the mailing date of this communication to copy this patent claim. Failure to do so will be considered a concession that the subject matter of this claim is the prior invention of another under 35 U.S.C. 102(g), and thus also prior art under 35 U.S.C. 103(a) (*In re Oguie*, 517 F.2d 1382, 186 USPQ 227 (CCPA 1975)), but will **not** result in the abandonment of this application. THE PROVISIONS OF 37 CFR 1.136 DO **NOT** APPLY TO THE TIME SPECIFIED IN THIS ACTION.

**Examiner Note**

1.
In bracket 1, insert the number from the patent of the suggested claim.
2.
In bracket 2, insert the number of the patent.
3.
In bracket 3, insert a copy of the patent claim.
4.
Only one claim from the patent should be suggested for interference unless other claims to a separate patentably distinct invention are claimed in the patent and can be made by the applicant. To suggest an additional claim, form paragraph 23.10 should follow this paragraph.
5.
If the Office action addresses other issues, such as a rejection of other claims, form paragraph 23.07 should be included at the end of the Office action.

¶ *23.10 Copying Additional Patent Claims for a Distinct Invention*

Claim number **[ 1 ]** from U.S. Patent No. **[ 2 ]** is suggested under 35 U.S.C. 135(a) in addition to claim **[ 3 ]** of the patent, suggested above. The inventions defined by these patent claims are considered to be "separate patentable inventions" under 37 CFR 1.601(n) which could form the basis for plural counts in an interference.

The suggested claim, reproduced below, must be copied exactly, although other claims may be proposed under 37 CFR 1.605(a).

**[ 4 ]**

Applicant is given ONE MONTH or THIRTY DAYS, whichever is longer, from the mailing date of this communication to copy this additional patent claim. Failure to do so will be considered a concession that the subject matter of this claim is the prior invention of another under 35 U.S.C. 102(g), and thus also prior art under 35 U.S.C. 103(a) (*In re Oguie*, 517 F.2d 1382,186 USPQ 227 (CCPA 1975)). THE PROVISIONS OF 37 CFR 1.136 DO **NOT** APPLY TO THE TIME SPECIFIED IN THIS ACTION.

**Examiner Note**

1.
In bracket 1, insert the number of the patent claim that is patentably distinct from the claim specified in form paragraph 23.09.
2.

AJP - Cell Physiology


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# Regulation by retinoids of $P2Y_2$ nucleotide receptor mRNA in human uterine cervical cells

**George I. Gorodeski[1,2], Peter Burfeind[1], Shu Uin Gan[1], Dipika Pal[1], and Fadi W. Abdul-Karim[3]**

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## ABSTRACT

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Extracellular ATP stimulates acute changes in paracellular permeability across cultures of human uterine cervical epithelial cells [G. I. Gorodeski, D. E. Peterson, B. J. De Santis, and U. Hopfer. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1715-C1725, 1996]. In this paper, we characterize mRNA for a $P2Y_2$ nucleotide receptor in human cervical cells. Using oligonucleotide primers based on the sequence of human airway epithelium $P2Y_2$ receptor, a single 632-bp cDNA band was identified in RT-PCR experiments in extracts of human endocervical and ectocervical tissues and in lysates of human cervical CaSki cells, but not in 3T3 fibroblasts. The nucleotide sequence was homologous to the corresponding human airway epithelium $P2Y_2$ receptor. Northern blot analyses revealed hybridization of the $P2Y_2$ receptor probe to a 2.0-kb mRNA fragment, as well as to 2.2-, 3.0-, and 4.6-kb species, indicating that human cervical cells express $P2Y_2$ receptor mRNA. Incubation of CaSki cells in retinoid-free medium abolished the ATP-induced changes in permeability and decreased the expression of the $P2Y_2$ receptor mRNA; treatment with retinoids restored the responses to ATP and upregulated the $P2Y_2$ receptor mRNA, suggesting that the receptor mediates ATP-related changes in permeability. Treatment with actinomycin D decreased the expression of the $P2Y_2$ receptor RNA, but the ratio density of the receptor RNA relative to glyceraldehyde-3-phosphate dehydrogenase RNA remained unchanged, suggesting that retinoids upregulate transcription of the receptor mRNA. We conclude that retinoid-dependent modulation of the $P2Y_2$ receptor expression, and hence of the responses to ATP, may be an important mechanism for the regulation of secretion of cervical mucus in vivo.

extracellular adenosinetriphosphatase; $P_{2U}$ and $P_{2N}$ nucleotide receptors; vitamin A; paracellular permeability; transepithelial transport; cervical mucus

# INTRODUCTION

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THE MAIN FUNCTION OF THE epithelium that covers the woman's uterine cervix is to regulate secretion of fluid and solutes, the cervical mucus, into the cervical canal. Recently, we described an in vitro model of human cervical epithelial cells that retains phenotypic characteristics of the endocervical epithelium (24). The cultured cervical epithelium is characterized by a relatively high degree of permeability (14), suggesting that transcervical transport occurs mainly via the paracellular pathway.

These studies also showed that micromolar concentrations of extracellular purines (ATP) or pyrimidines (UTP) modulate the paracellular permeability across cervical cultures acutely and reversibly (19, 23) and that the effects could be described in terms of activation of nucleotide receptors (20). In keeping with the latest nomenclature proposal, the results suggested activation of $P2Y_2$ nucleotide receptors (1, 6, 7). These novel data may be relevant to the regulation of cervical mucus in vivo because epithelial cells express $P2Y_2$-nucleotide receptors (7, 8, 27, 30, 31) and ATP can accumulate in the extracellular space at micromolar concentrations (5, 11) that are sufficient to activate the $P2Y_2$ receptor mechanism. Our first objective in the present study was to determine whether human cervical cells express nucleotide receptor mRNA.

Vitamin A and related compounds, the retinoids, regulate the reproductive tract epithelia in females (9, 12, 15, 17, 18). Vitamin A deficiency results in dryness of mucous membranes (9) and may lead to squamous metaplasia and keratinization of the simple columnar epithelium of the endocervix (12). In addition, retinoids regulate the paracellular permeability across the cultured human cervical epithelium (15) and modulate the responses to extracellular ATP (22). Our second objective was to test the hypothesis that in human cervical cells the effects of extracellular ATP are mediated by nucleotide receptor and that retinoids modulate the responses to extracellular ATP by regulating the expression of the $P2Y_2$ receptor. We characterized mRNA for a $P2Y_2$ receptor in human endocervical and ectocervical tissues and in cultured human cervical epithelial cells. Our results also show that the expression of the receptor mRNA is regulated by retinoids, both retinoic acid receptor (RAR)-selective and retinoid X receptor (RXR)-selective agonists, and that changes in the cellular density of the $P2Y_2$ mRNA receptor correlate with the responses to ATP.

# METHODS

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*Collection of endocervical and ectocervical tissues.* Endocervical and ectocervical tissues were obtained from uteri of premenopausal women who underwent hysterectomy for indications unrelated to the study and had histologically normal cervices. After removal of the uterus, the cervical tissues were washed, minced, and transferred to the lab in ice-cold saline.

*Cell culture techniques.* CaSki cells, which retain phenotypic characteristics of human endocervical cells (1), were grown and maintained on culture dishes in regular medium enriched with 8% FCS as was described (14, 19, 20, 23, 24). In some experiments, cells were shifted to a medium enriched with FCS that was delipidized (retinoid-free medium) (22). 3T3 fibroblasts were cultured as described (17, 18).

*Electrophysiological experiments.* Changes in paracellular resistance were determined in terms of changes in the

transepithelial electrical conductance ($G_{TE}$) as described (23). Changes in $G_{TE}$ were calculated continuously across filters mounted in a modified Ussing chamber from successive measurements of the short-circuit current ($I_{sc}$) and the transepithelial potential difference (PD), switching between $I_{sc}$ and PD at a rate of 20 Hz: $G_{TE} = \Delta I_{sc} / \Delta PD$. The experimental design of the electrophysiological measurements, including calibrations and controls, the significance of the voltage and $I_{sc}$, and the conditions for optimal determinations of $G_{TE}$ across the low-resistance CaSki epithelium were described and discussed (15, 23).

*Isolation of RNA.* We used well-established methods (4) with some modifications (16, 17). Tissues (endocervix and ectocervix) were pulverized and then homogenized by tissue tearer using the Qiagen kit (Qiagen, Chatsworth, CA) with lysis buffer plus 2-mercaptoethanol at 350 μl/30 mg. Total RNA from CaSki cells or from 3T3 fibroblasts was isolated with the Qiagen kit, using lysis buffer plus 2-mercaptoethanol at 350 μl/$10^7$ cells. The final total RNA pellets were resuspended in 50 μl of diethyl pyrocarbonate water and quantitated by measuring the optical density at 260 nm. Total RNA of human placenta was a generous gift from Dr. J. Ilan (Department of Reproductive Biology, Case Western Reserve University School of Medicine, Cleveland, OH). Poly(A)$^+$ RNA was isolated from total RNA using an oligo (dT) cellulose affinity column (Poly[A] Quik column, Stratagene, La Jolla, CA), according to the manufacturer's protocol. For Northern blots, we used 20 μg total RNA and 7-10 μg poly(A)$^+$.

RT-PCR was carried out in a Perkin-Elmer DNA thermal cycler (Cetus, Norwalk, CT) using an RT-PCR kit (Boehringer Mannheim, Indianapolis, IN). Total RNA (1.5 μg), denatured at 65°C for 5 min, was reverse transcribed in a final volume of 20 μl of reaction mixture containing 10 mM Tris · HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dNTPs, 5 μM oligo$(dT)_{15}$ (Promega, Madison, WI), 40 units RNase inhibitor, and 25 units avian myeloblastosis virus (AMV) RT (Boehringer Mannheim, Mannheim, Germany). For mock reaction, a similar tube was added without the oligo(dT) and without the AMV RT. The reaction was allowed to proceed at 42°C for 60 min and was terminated by heating to 99°C for 2 min. The sample was diluted 1:5 with double-distilled $H_2O$. PCR was then performed in a 50-μl volume using 5 μl of the diluted sample, 5× PCR buffer (Amersham, Arlington Heights, IL), each primer at 1 μM, 0.01 mM dNTPs, 5 units *Taq* polymerase (Amersham), and 1.4 μM *Taq* antibody (Clonetech) in a Perkin-Elmer 460 DNA thermal cycler (Cetus). The following conditions were applied: 94°C for 5 min, followed by 30 cycles of 1 min of denaturation step at 94°C, 1 min of annealing step at 60°C, and 1 min of extension step at 72°C, followed by 7 min at 72°C. Samples were cooled at 4°C (soak file) and frozen at −80°C to facilitate removal of the mineral oil. The primers used to amplify the $P2Y_2$ receptor were synthesized by the Molecular Biology Core Laboratory of the Case Western Reserve University School of Medicine and were prepared as 10 μM stocks. The following oligonucleotide primers were used, based on the reported corrected sequence of a $P_{2U}$ nucleotide receptor cloned from human airway epithelial cells (CF/T43) (32): forward (sense), 5'-CTC TAC TTT GTC ACC ACC AGC GCG-3' (nucleotides 750-773); and reverse (antisense), 5'-TTC TGC TCC TAC AGC CGA ATG TCC-3' (nucleotides 1364-1387). The sequences used to amplify the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment were GAPDH forward (sense), 5'-TGA AGG TCG GAC TCA ACG GAT TTG GT-3'; and GAPDH reverse (antisense), 5'-GTG GTG GAC CTC ATG GCC CAC ATG-3'. Amplified samples (20 μl) were analyzed on 1.5% agarose gel, stained with ethidium bromide, and photographed. Densitometry of bands was taken from the Polaroid negatives using an Ultrascan XL laser densitometer (Pharmacia). Parallel experiments were routinely done using DNase I before RT, to exclude amplification of genomic cDNA contaminants. The DNA molecular weight markers were from *Hinc* II digest of OX174 DNA

(United States Biochemical, Cleveland, OH).

*Northern blot analysis.* Total RNA or mRNA was separated on a 1% denaturing agarose gel, transferred to a Hybond N nylon membrane (Amersham, Cleveland, OH), and cross-linked by brief exposure to ultraviolet light or baked at 80°C. The cDNA probes of the $P2Y_2$ receptor (see below) or of GAPDH (Clontech Laboratories, Palo Alto, CA) were labeled with [$^{32}$P]dCTP (NEN, Boston, MA) using the random hexanucleotide primer method and hybridized to Northern blots in 5× standard saline citrate (SSC), 5× Denhardt's solution, 10% (wt/vol) dextran sulfate, 0.1% (wt /vol) SDS, and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 h. The filter was washed at room temperature for 15 min in 2× SSC, followed by 5-15 min in 0.5× SSC and 0.5% (wt /vol) SDS at 65°C. The filter was exposed to X-ray film for 6-24 h.

*Densitometry.* The X-ray films were analyzed with a laser densitometer (Sciscan 5000, United States Biochemical) and normalized to GAPDH RNA.

*Cloning and sequencing of the partial $P2Y_2$ nucleotide receptor PCR fragment.* RT-PCR of CaSki cell total RNA using oligonucleotide primers specific to the $P_{2U}$ nucleotide receptor of human airway epithelial cells (32) yielded a single 632-bp fragment. The fragment was extracted from a 1% agarose gel, purified using the Qiagen gel extraction kit (Qiagen) according to the manufacturer's protocol, and cloned into the *Hinc* II site of the pGEM3z vector (Promega). Before transformation of DH5α cells, plasmid DNA was prepared from single colonies using a miniprep kit (Promega) with the manufacturer's procedure. The subcloned partial $P2Y_2$ nucleotide receptor DNA sequence was then sequenced in both directions using the $P_{2U}$ forward and reverse primers described in the previous section. Sequencing was performed by the Molecular Biology Core Laboratory at Case Western Reserve University, using an ABI373A automated sequencer. The sequences were checked against the GenBank databases using the National Center for Biotechnology Information BLAST network services and was confirmed to be homologous to $P2Y_2$ nucleotide receptor (accession no. U07225/HSU07225).

*Statistical analysis of data.* Data are presented as means ± SD, and significance of differences among means was estimated by Student's *t*-test. Trends were calculated using GB-STAT V5.3 (1995; Dynamic Microsystems, Silver Spring, MD) and analyzed with ANOVA.

*Chemicals and supplies.* Anocell (Anocell-10) filters were obtained from Anotec (Oxon, UK). 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl)-1-propenyl]benzoic acid (TTNPB), SRI-11217, and SRI-11237 were synthesized in the Department of Chemistry, Allergen (Irvine, CA), and were a gift of Dr. R. Eckert (Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, OH). TTNPB is a potent synthetic RAR-selective retinoid, whereas SRI-11217 and SRI-11237 are RXR-selective synthetic retinoids (3). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical (St. Louis, MO). All retinoids were prepared as 1,000× stocks in DMSO and stored in the dark at −20°C.

# RESULTS

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*Effects of retinoids on phase I and phase II responses to extracellular ATP and to other nucleotides.* Extracellular ATP (50 μM) stimulates a biphasic change in $G_{TE}$ across CaSki cells, which is the

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composite of two effects (19, 23): an acute increase in the permeability of the lateral intercellular space (phase I response) and a subsequent, slower decrease of the tight junctional permeability (phase II response) (Fig. 1*A*). In cells incubated in retinoid-free medium, baseline $G_{TE}$ decreased, and the responses to ATP were abrogated (Fig. 1*B*). Treatment of cells with 10 nM all-*trans*-retinoic acid (tRA) restored baseline $G_{TE}$ and the phase I response to ATP (Fig. 1*C*); the amplitude of phase II response was restored only in part compared with cells grown in regular medium (Fig. 1, *A* and *C*). These results indicate that retinoids modulate the ATP-induced changes in permeability.

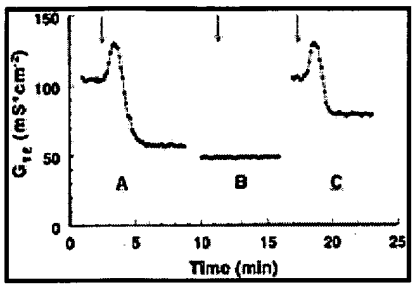


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**Fig. 1.** Effects of retinoids on changes in transepithelial electrical conductance ($G_{TE}$) across cultures of CaSki cells on filters in response to extracellular ATP. When indicated (arrows), ATP was added at a concentration of 50 μM to luminal and subluminal solutions from 1,000× concentrated stock (pH 7.2). Shown are tracings of changes in $G_{TE}$. *A*: CaSki cells grown in regular medium. Early increase in $G_{TE}$ is phase I response, and late decrease in $G_{TE}$ is phase II response. *B*: cells were grown for 5 days in retinoid-free medium. *C*: cells were grown for 3 days in retinoid-free medium and then treated with 10 nM all-*trans*-retinoic acid (tRA) for 2 additional days. Experiments were repeated 4 times.

The effects of extracellular ATP on $G_{TE}$ across CaSki cells could be described in terms of activating $P2Y_2$ nucleotide receptors (20). To determine whether retinoids also modulated the responses to other nucleotides, we repeated the experiment also with UTP, ATPγS, ADP, and AMP. In control cells grown in regular medium, some of these agents produced acute changes in $G_{TE}$, but the effects differed in magnitude compared with ATP: in general, the order of efficacy was ATP ≈ UTP > ATPγS > ADP and AMP, similar to our previous report (20). Incubation of cells in retinoid-free medium abolished the responses to all agonists, whereas treatment with 10 nM tRA restored the phase I responses and, in part, the amplitude of phase II responses (Table 1). These results indicate that retinoids also modulate the changes in permeability in response to other ligands of the $P2Y_2$ receptor.

**Table 1.** Retinoid effects on agonist-induced changes in $G_{TE}$ across CaSki cultures

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*Characterization of $P2Y_2$ nucleotide receptor mRNA in human cervical cells.* With the use of oligonucleotide primers complementary to a $P_{2U}$ nucleotide receptor cloned from human airway epithelial cells (CF/T43) (32), a single cDNA fragment (632 bp) was amplified by RT-PCR from human CaSki cells (Fig. 2*A*). Similar results were obtained in tissues of human endocervix and ectocervix but not in extracts of 3T3 fibroblasts (Fig. 2*A*). Polyacrylamide gel analysis of this product revealed a single band of 632 bp (Fig. 2*B*), which corresponded to the expected length based on the predicted sequence of the partial length of the $P_{2U}$ nucleotide receptor of human airway epithelial cells (32). This

cDNA fragment was isolated, amplified, and purified, and the product was sequenced by the dideoxy chain termination method. Sequence analysis of the cloned segment revealed homologies of 98% (sense) and 96% (antisense) with the human airway epithelium $P_{2U}$ nucleotide receptor (32) (the differences were sequence errors; not shown).

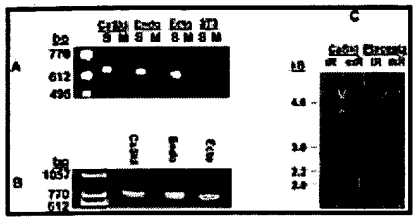

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**Fig. 2.** Characterization of $P2Y_2$ nucleotide receptor mRNA in human cervical cells. *A* and *B*: RT-PCR results using total RNA extracted from CaSki cells, from tissues of human endocervix (Endo) and ectocervix (Ecto), and from 3T3 fibroblasts. Oligonucleotide primers complementary to a $P_{2U}$ nucleotide receptor cloned from human airway epithelial cells were used to amplify a single cDNA fragment of 632 bp. Samples were analyzed on either agarose gels (*A*) or polyacrylamide gels (*B*). S, sample; M, mock reaction [excluding oligo(dT) and avian myeloblastosis virus RT; see METHODS]. *C*: total RNA (tR) or poly(A)$^+$ RNA (mR) extracts of CaSki cells or of human term placenta were separated on Northern gels and hybridized with radiolabeled cDNA of partial-length $P2Y_2$ receptor. Probe hybridized to total RNA and mRNA species of 4.6, 3.0, 2.2, and 2.0 kb (CaSki) and of 4.6 and 2.0 kb (placenta). Experiments were repeated 3-6 times. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Expression of the $P2Y_2$ mRNA receptor in human CaSki cells was also analyzed by hybridization of the cloned segment of the $P2Y_2$ receptor to total RNA and mRNA. Northern analyses revealed that the radiolabeled cDNA of the partial-length $P2Y_2$ receptor hybridized to total RNA and mRNA species of 2.0, 2.2, 3.0, and 4.6 kb (Fig. 2*C*; see also Figs. 5 and 6). The 2.0-kb band was most abundant, whereas the other three were present at lower levels, and their relative expression differed among the experiments. The 2.0- and 4.6-kb species, and perhaps the 2.2-kb species, were also present in total RNA and mRNA of human placenta (Fig. 2*C*). Collectively, these results indicate that human cervical cells express a $P2Y_2$ receptor mRNA.

*Regulation by retinoids of $P2Y_2$ nucleotide receptor mRNA in human cervical cells.* Treatment of CaSki cells with 10 nM tRA for 2 days had no effect on the expression of GAPDH mRNA, but it increased the expression of the $P2Y_2$ receptor mRNA (the 632-bp band in RT-PCR assays; Fig. 3). Results of four densitometry experiments revealed that expression of the $P2Y_2$ receptor mRNA relative to that of GAPDH mRNA increased 2.5 ± 0.4-fold with the addition of tRA, compared with control cells.

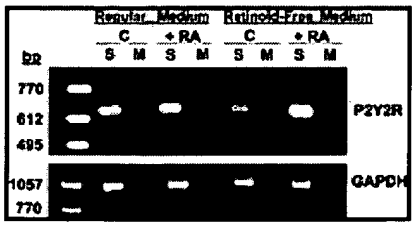


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**Fig. 3.** Effects of retinoids on expression of $P2Y_2$ receptor mRNA in CaSki cells: RT-PCR results. Two days after plating, cells were grown for 3 days in regular medium or in retinoid-free medium and then treated either with 10 nM tRA (+RA) or with vehicle (C; control) for 2 additional days. Experiments were repeated 4 times.

To better understand the effect of retinoids on $P2Y_2$ mRNA receptor expression, cells were cultured for 3 days in retinoid-free medium and then treated with tRA for 2 additional days. Neither incubation in retinoid-free conditions nor treatment with tRA had any significant effect on the GAPDH mRNA (Fig. 3). In contrast, retinoids modulated the expression of the $P2Y_2$ receptor mRNA. In cells grown in retinoid-free medium, the ratio expression of $P2Y_2$ receptor mRNA to GAPDH mRNA decreased, significantly, 3.3 ± 0.4-fold compared with that in cells incubated in regular medium (Fig. 3). Treatment of cells cultured in retinoid-free medium with tRA increased the density of the $P2Y_2$ receptor mRNA significantly: the ratio of $P2Y_2$ receptor mRNA to GAPDH mRNA increased 10.2 ± 0.4-fold compared with cells grown in retinoid-free medium and by 3.5 ± 0.4-fold compared with cells grown in regular medium and not treated with tRA (Fig. 3).

To ascertain that the RT-PCR technique is sensitive in measuring changes in the expression of the $P2Y_2$ receptor, the experiment was repeated using different amounts of cDNA for the PCR amplification. As is shown in Fig. 4 and summarized in Table 2, the quantity of the amplified products of both the GAPDH and the $P2Y_2$ receptor cDNA was dependent on the amount of cDNA used for the amplification. Treatment with tRA had no effect on GAPDH RNA, but it increased $P2Y_2$ receptor RNA (Fig. 4). Subsequently, and regardless of the amount of initial cDNA used for the PCR, the ratio of $P2Y_2$ receptor RNA to GAPDH RNA increased following treatment with tRA by ~4.5-fold compared with retinoid-free conditions (Table 2).

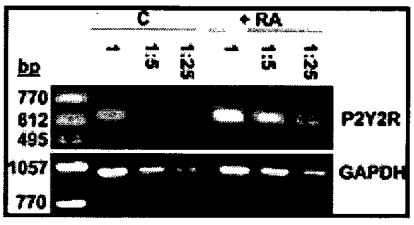


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**Fig. 4.** Effects of retinoids on expression of $P2Y_2$ receptor mRNA in CaSki cells: RT-PCR results. Two days after plating, cells were shifted to retinoid-free medium and then treated either with 10 nM tRA (+RA) or with vehicle (C; control) for 2 additional days. For PCR, 375 ng (1), 75 ng (1:5), and 15 ng (1:25) of cDNA were used in each group, both for $P2Y_2$ receptor cDNA and for GAPDH. Experiment was done twice, and results are summarized in Table 2.

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**Table 2.** Retinoid effects on expression of $P2Y_2$ receptor mRNA in CaSki cells: RT-PCR experiments

To confirm these findings, we used Northern analyses to study the effects of retinoids on the expression of the $P2Y_2$ receptor total RNA. Incubation in retinoid-free medium decreased the density of the 2.0-, 2.2-, 3.0-, and 4.6-kb bands but did not have a significant effect on the levels of GAPDH total RNA (Figs. 5 and 6). Densitometry of the bands revealed that incubation in retinoid-free medium decreased the 2.0-, 2.2-, and 4.6-kb products relative to GAPDH by 80-90% (Table 3). The 3.0-kb product was minimally expressed and therefore it was difficult to quantify the changes (Fig. 5). Treatment with tRA upregulated the 2.0-, 2.2-, and 4.6-kb bands to levels observed in cells incubated in regular medium (Figs. 5 and 6, Tables 3 and 4).

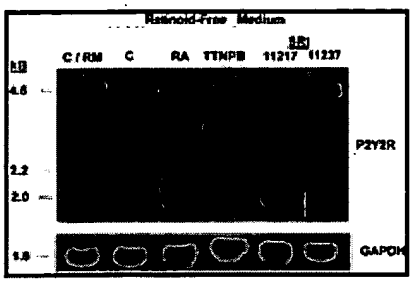


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**Fig. 5.** Effects of retinoids on expression of $P2Y_2$ receptor RNA in CaSki cells: Northern analysis. Two days after plating, cells were grown for 3 days in regular medium (C/RM) or in retinoid-free medium and then treated with vehicle (C; control) or with 10 nM of 1 of following retinoids for 2 additional days: tRA (RA), TTNPB, SRI-11217, or SRI-11237. Experiment was done twice, and results are summarized in Table 3.

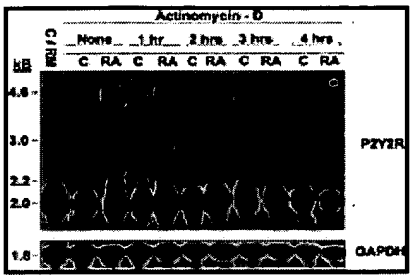


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**Fig. 6.** Effects of retinoids and of actinomycin D on expression of $P2Y_2$ receptor RNA in CaSki cells: Northern analysis. Two days after plating, cells were grown for 5 days in regular medium (C/RM) or shifted to retinoid-free medium for 3 days. Latter were then treated with 10 nM of tRA (RA) or with vehicle (C; control) for 2 additional days. Actinomycin D (10 μM) was added for 1-4 h before extraction of RNA. Experiment was done twice.

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**Table 3.** Retinoid effects on expression of $P2Y_2$ receptor mRNA in CaSki cells: Northern blot experiments

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**Table 4.** Effects of retinoic acid and of actinomycin-D on expression of 2.0-kb band of $P2Y_2$ receptor mRNA in CaSki cells: Northern blot experiments

*Role of RAR and of RXR.* To study the specificity of retinoid effects on the $P2Y_2$ receptor RNA, we compared the effects of tRA to those of other retinoids, all added at a concentration of 10 nM. TTNPB, an RAR-selective ligand, and SRI-11217 and SRI-11237, RXR-selective ligands, upregulated the 2.2- and 4.6-kb bands significantly and upregulated the 2.0-kb bands nearly to the control levels observed in cells grown in regular medium (Fig. 5, Table 3). Collectively, the results shown in Figs. 3-6 indicate that incubation of CaSki cells in retinoid-free medium decreases the density of the $P2Y_2$ receptor mRNA and that agonists of both the RAR and RXR receptors can upregulate the $P2Y_2$ receptor mRNA.

*Mechanisms of the effects of retinoids.* Retinoids modulate cell functions via several known mechanisms, including

transcription regulation (13). The objective of the next experiment was to study the effects of the transcription inhibitor actinomycin D on the tRA-induced expression of the $P2Y_2$ receptor RNA. Cells were shifted to retinoid-free medium for 3 days and then treated with 10 nM tRA or the vehicle (control) for 2 additional days. Actinomycin D (10 μM) was added for 1-4 h before extraction of total RNA. Treatment with actinomycin D decreased the expression of the 2.0-, 2.2-, 3.0-, and 4.6-kb bands of the $P2Y_2$ receptor RNA, and of the GAPDH RNA, in a time-related manner, both in control cells and in cells treated with tRA (Fig. 6). Because the expression of the 2.2-, 3.0-, and 4.6-kb species was weak, we considered changes only in the 2.0-kb band for densitometric analysis. As is shown in Fig. 6 and summarized in Table 4, treatment with actinomycin D decreased the expression of the 2.0-kb transcript of the $P2Y_2$ receptor RNA, and of the GAPDH RNA, with time. However, the density of the 2.0-kb band relative to GAPDH did not change over the 4-h period of incubation with actinomycin D either in control cells or in cells that were treated with tRA (Fig. 6, Table 4).

# DISCUSSION

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Extracellular ATP and UTP stimulate acute changes in paracellular permeability across cultured human cervical epithelia (Ref. 19 and present study), and the responses can be modulated by retinoids (22). Previous studies (20, 21), using pharmacological criteria, described the effects in terms of activating $P2Y_2$ receptor(s) [formerly designated $P_{2U}$ or $P_{2N}$, (1, 6, 7)]. The present results describe, for the first time, the expression and regulation by retinoids of a nucleotide receptor mRNA in human cervical epithelial cells. On the basis of the experimental findings, we suggest that the newly described receptor mediates the ATP- and UTP-induced changes in transcervical paracellular permeability.

Cervical cells expressed at least four distinct transcripts that cross-hybridized with the partial-length $P2Y_2$ receptor cDNA, but the most abundant was the 2.0-kb transcript. Other human tissues also express a number of different mRNA for the $P2Y_2$ receptor (32), but no definitive conclusion was reached as to whether these are unprocessed or alternatively spliced forms of the receptor mRNA or whether they are products of distinct genes. The relative abundance of the 2.0-kb band, the variability in the expression of the 2.2-, 3.0-, and 4.6-kb transcripts among different experiments, and the parallel changes in all four bands in response to vitamin A deprivation and to retinoid treatment suggest that in CaSki cells the different bands represent intermediary steps in receptor mRNA processing. However, the possibility that these bands are alternatively processed forms of the same gene, or products of different genes, has not been ruled out and is currently being studied.

Despite the important role that nucleotide receptors may have for cell function in vivo, only a few studies have focused on regulation of the responses to extracellular nucleotides and of the cellular content of the nucleotide receptors. Hung et al. (26) reported that treatment of chondrocytes isolated from chick embryo sternum with nanomolar concentrations of retinoic acid stimulated responsiveness to ATP in the form of increases in cytosolic calcium. The authors hypothesized that retinoic acid upregulated a nucleotide receptor and that the effect plays a role in chondrocyte differentiation. Martin et al. (29) reported that phorbol esters or inflammatory activators downregulate $P_{2U}$ nucleotide receptor mRNA expression during in vitro differentiation of human myeloid leukocytes.

Retinoids modulate changes in paracellular permeability across human cervical cells induced by extracellular ATP. It

was suggested that retinoids modulate a proximal step (or steps) in the ATP-dependent stimulus (22), possibly the ATP receptor. The present results support this hypothesis and indicate that retinoids control the expression of $P2Y_2$ receptor mRNA in CaSki cells. Incubation of cells in retinoid-free medium decreased the expression of the $P2Y_2$ receptor mRNA, whereas treatment with physiological levels of retinoic acid increased it. These results correlated with the effects of retinoids on paracellular permeability, namely that incubation in retinoid-free medium abrogated the phase I and phase II responses to ATP and UTP, whereas treatment with retinoic acid restored the effects (present results and Ref. 22). These findings suggest that the retinoid-induced changes in permeability are the result of modulation of the $P2Y_2$ receptor expression.

The effects of retinoids on gene expression are mediated via interaction with the nuclear retinoid receptors. The nuclear retinoid receptor family consists of six members, RAR α, β, and γ and RXR α, β, and γ (13), that function as ligand-activated *trans*-acting factors. RAR and RXR receptors are expressed in human cervical cells (2, 3, 25), and their cellular levels can be modulated by retinoids (3). The effects of retinoids on the transcervical permeability responses to ATP are also mediated via the retinoid receptor mechanism (15, 22). The present results extend these findings and indicate that both RAR-selective ligands (e.g., TTNPB) and RXR-selective ligands (e.g., SRI-11217 and SRI-11237) can upregulate the $P2Y_2$ receptor. Collectively, these findings suggest that the retinoid regulation of the $P2Y_2$ receptor and the responses to ATP are downstream from the retinoid receptor(s). RAR-selective ligands are important regulators of gene expression and differentiation in cervical cells (2, 25). In contrast, RXR ligands have been shown to be relatively inactive (3, 25). This does not appear to be the case for the effects on paracellular resistance (15), on the ATP-induced increase in permeability (22), and on the expression of the $P2Y_2$ receptor (present study), as SRI-11217 and SRI-11237 (selective ligands of the RXR) were effective regulators in CaSki cells. Our results therefore suggest a role for RXR-specific receptors in regulation of cervical cell nucleotide receptors and paracellular permeability.

One of our objectives was to study the mechanisms by which retinoids modulate expression of the $P2Y_2$ receptor. In cells incubated in retinoid-free medium and treated with tRA, actinomycin D decreased significantly the expression of both the $P2Y_2$ receptor RNA and the GAPDH RNA, but the density of the $P2Y_2$ receptor relative to GAPDH did not change significantly. These results support the hypothesis that retinoids upregulate $P2Y_2$ receptor mRNA by stimulating transcription rather than by increasing the stability of the message.

Nucleotide receptor regulation of paracellular permeability and the effects of retinoids may be important for understanding regulation of transcervical transport in vivo, because even small changes in transepithelial permeability may have profound effects on secretion of fluid and solutes and subsequently on the production of cervical mucus (14). Under baseline conditions, the extracellular levels of ATP are too low to effectively modulate transcervical paracellular resistance. However, under certain conditions, ATP can reach micromolar concentrations that are sufficient to activate the cervical cell $P2Y_2$ receptor and to modulate permeability (5, 11). These include sudden breakage of intact cells, exocytotic secretion of ATP from nerve endings or from nonneuronal cells (such as platelets, mast cells, and basophilic leukocytes), release of ATP from T lymphocytes following stimulation of cytolysis-dependent pore-forming proteins, release of ATP from the putative ATP-binding cassette ("ABC") transporters (possibly the multidrug resistance gene; Ref. 11), and release of ATP in seminal fluid after intercourse (28). Cervical epithelial cells therefore can be exposed to episodic release of ATP into the extracellular space, which can activate the $P2Y_2$ receptor, modulate paracellular

permeability, and affect transcervical transport.

Our findings that retinoids modulate transcervical permeability and the expression of $P2Y_2$ receptor in cervical cells may be important for the overall understanding of cervical mucus production in vivo. Vitamin A deficiency leads to dryness of mucous membranes, including the endocervix in women (9, 10, 12). According to our current understanding of transepithelial transport, these phenomena are the result of diminished transudation of fluids from the blood into the lumen across the cervical epithelium. In contrast to vitamin A deficiency, treatment with vitamin A or with retinoids reverses the condition (9, 10, 12). Retinoids increase baseline transcervical permeability (15) and upregulate the ATP-induced increase in permeability (22), possibly as a result of increasing the $P2Y_2$ receptor (present results). This would tend to increase the permeability and augment secretion of cervical fluid. Retinoids also upregulate the ATP-induced late decrease in permeability (22), a response that may have an opposite effect on cervical mucus. However, the latter effect is less sensitive to retinoids than the ATP-related increase in permeability (22), and the overall effect of retinoids would therefore tend to increase transport across the cervical epithelium.

Plasma levels of vitamin A in the human are tightly regulated (9, 12), and changes in cervical permeability in vivo are unlikely to be determined by changes in plasma levels of vitamin A per se. Possible explanations are changes in the rate of vitamin A conversion to more active metabolites (e.g., tRA) or the status of tissue retinoid receptors. The present results suggest an additional mechanism, notably regulation of transcription of $P2Y_2$ receptor mRNA and modulation of the expression of the $P2Y_2$ receptor in cervical cells.

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## FOOTNOTES

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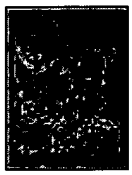

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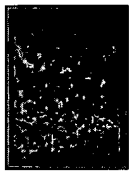

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